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Quantitative Analysis of Erythrocytes Containing Fetal Hemoglobin (F Cells) in Children With Sickle Cell Disease

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Variation in the level of fetal hemoglobin (HbF) accounts for much of the clinical heterogeneity observed in patients with sickle cell disease (SCD). The HbF level has emerged as an important prognostic factor in both sickle cell pain and mortality, and a % HbF of 10–20% has been suggested as a threshold level for diminished clinical severity. The number of erythrocytes that contain HbF (termed F cells) may also be critically important, as F cells resist intravascular sickling and have preferential in vivo survival. Since F cells can be enumerated with high accuracy using flow cytometry methods, we prospectively studied a cohort of 242 children with SCD. Children with HbS and hereditary persistence of fetal hemoglobin (S/HPFH) had essentially 100% F cells. In contrast, children with homozygous sickle cell anemia (HbSS), HbS/β^0 thalassemia, or HbS/β^+ thalassemia had significantly lower mean % F cell values (55.9, 61.6, and 51.3%, respectively; $P < 0.001$), and children with HbSC had even fewer F cells (27.0%; $P < 0.001$). There was a highly significant correlation between the % F cells and the log (% HbF), which was observed for the total population of children ($r = 0.95$, $P < 0.001$), as well as for each of the individual subgroups of children with HbSS ($r = 0.94$, $P < 0.001$), HbSC ($r = 0.89$, $P < 0.001$), or HbS/β^0 thalassemia and HbS/β^+ thalassemia ($r = 0.95$, $P < 0.001$). This logarithmic correlation between % F cells and % HbF has not been previously described and has important implications for the pharmacologic manipulation of HbF in patients with SCD. Am. J. Hematol. 54:40–46, 1997

Key words: children; erythrocytes; F cells; fetal hemoglobin; sickle cell disease

INTRODUCTION

The clinical manifestations of sickle cell disease (SCD) result from chronic hemolytic anemia and the effects of intravascular sickling, which include local tissue hypoxia and organ damage [1,2]. Patients with SCD commonly develop painful vaso-occlusive events and have an increased risk of infection secondary to splenic infarction. Other common clinical events include acute chest syndrome, splenic sequestration, avascular necrosis, cholelithiasis, priapism, and cerebral vascular accident. There is a large clinical heterogeneity observed in SCD, even for patients with an identical hemoglobin phenotype [3–5]. This variation in clinical severity can be explained in part by differences in the total hemoglobin concentration, the mean corpuscular hemoglobin concentration, erythrocyte rheology, the percentage of adhesive cells, the proportion of dense cells, the presence or absence of α-thalassemia, and the β-globin haplotype [3,6–9].

The percentage of fetal hemoglobin (% HbF), however, is perhaps the most important laboratory parameter influencing clinical severity in SCD [10,11]. In normal individuals, HbF comprises only 5% of the total hemoglobin by age 3–6 months and falls to below 1% in adults [12]. In contrast, patients with SCD typically have HbF levels ranging from 1 to 20% [13]; those with genetic mutations leading to hereditary persistence of fetal hemoglobin (HPFH) can have HbF levels that reach 30–40% of the

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total hemoglobin [14]. Several studies have demonstrated that an increased % HbF is associated with decreased clinical severity in SCD, using endpoints such as the number of painful events, transfusions, and hospitalizations [3,15,16]. Other studies have failed to show a protective effect of HbF, perhaps because the HbF levels were too low to provide protection [7,17]. A potential threshold of 10–20% HbF has been suggested, above which patients experience fewer clinical events [18]. The % HbF has also emerged as the most important predictor of early mortality in patients with SCD [10,19].

Patients with SCD have elevated HbF levels in part due to their increased rate of erythropoiesis [12], but multivariate statistical analysis has documented that age, gender, the presence of α-thalassemia, the β-globin haplotype, and an X-linked locus also influence the total % HbF within a given individual [5,20–22]. Except in the case of HPFH, HbF is not found in all erythrocytes, but rather is located in a subset known as HbF-containing cells or "F cells" [23,24]. In normal adults, the percentage of F cells ranges from 0.5 to 7%, while in patients with SCD, the % F cells has a much broader range [14,23]. In a few patients with sickle cell anemia tested serially, the % F cells was stable over time within an individual, although the % F cell value varied greatly among different patients [23]. Because F cells have a decreased tendency toward sickle formation, they tend to survive preferentially in the peripheral blood of patients with SCD [23]. The number of F cells, therefore, may be of equal or even greater importance than the absolute amount of HbF in influencing the clinical severity of an affected individual. To date, no prospective analysis of F cells in patients with sickle cell disease has been reported. We therefore quantitated the % F cells in a large cohort of children with SCD and correlated the results with other laboratory parameters. We identified a significant logarithmic correlation between % F cells and % HbF, which has important implications for the pharmacologic increase of HbF in patients with SCD.

MATERIALS AND METHODS

Patient Population

All patients received medical care at the Duke Pediatric Hematology Clinic within the Duke-UNC Comprehensive Sickle Cell Center. The diagnosis of a sickle hemoglobinopathy was established using standard hemoglobin electrophoresis techniques, and the % HbF was determined by alkali denaturation. The diagnosis of HbS/β^+ thalassemia was established by the presence of HbA (typically 20–25%) in an untransfused sample. The diagnosis of β^0 thalassemia was established by the presence of an elevated amount ($> 3.5\%$) of HbA_2 quantitated by column chromatography [25]. When possible, family studies were also performed. All patients in this study were at least 2 years of age, and no patient had been transfused within 4 months.

F Cell Enumeration

Quantitation of F cell percentage was performed using a slight modification of a previously described protocol [26]. Briefly, whole blood (0.3 mL collected in EDTA) was washed twice with borate saline. To crosslink and fix the erythrocytes, Dimethyl 3,3′-dithiobispropionimidate (DTBP, Pierce, Rockford IL) was added to 1 mL of washed cells, followed by cold borate saline containing diethanolamine. The fixed erythrocytes were resuspended in PBS/2% (phosphate-buffered saline) albumin and stored in 1 mL aliquots at −70°C until further analysis.

Fixed erythrocytes were thawed on ice and permeabilized using Triton X-100 and isopropanol, then aliquoted into two samples for immunophenotype analysis. One sample was incubated with a monoclonal antibody (mAb) specific for the γ-globin chain of fetal hemoglobin and has no reactivity with β-globin chains (Immuno-Rx, Atlanta GA). The other sample was incubated with a negative control mAb. After the primary mAb incubation, cells were washed with 0.8% Triton in PBS and then incubated with a goat anti-mouse $F(ab')_2$ conjugated to fluorescein (Pierce, Rockford IL). The cells were then washed twice and resuspended in 0.5 mL 0.8% Triton in PBS. Five thousand stained cells in each sample were analyzed using a FACSCAN flow cytometer (Beckton-Dickinson) and Lysis II software. Figure 1 shows six examples of F cell enumeration, on cord blood, normal erythrocytes, and samples from four children with sickle cell disease. Each histogram shows a distinct population of cells that stains positively for γ-globin and therefore represents F cells.

Statistical Analysis

The software program Primer (McGraw-Hill, New York, NY) was used for statistical analysis. Comparison of laboratory parameters with F cell quantitation was performed using linear regression, least squares analysis, ANOVA, and Student's paired *t* test.

RESULTS

Patient Population

A total of 242 pediatric patients were included in this study, including 134 males and 108 females. Table I shows that the majority of patients had homozygous sickle cell anemia (HbSS) ($n = 130$), followed by HbSC ($n = 74$), HbS/β^+ thalassemia ($n = 23$), HbS/β^0 thalassemia ($n = 7$), and HbS/HPFH ($n = 8$). For the entire group of children, the average age was 8.6 ± 4.8 years (mean $\pm$ 1 standard deviation; range, 2.0–19.5 years). There were no significant differences in the ages of children with each hemoglobin genotype (Table I).

The results of quantitative enumeration of % F cells

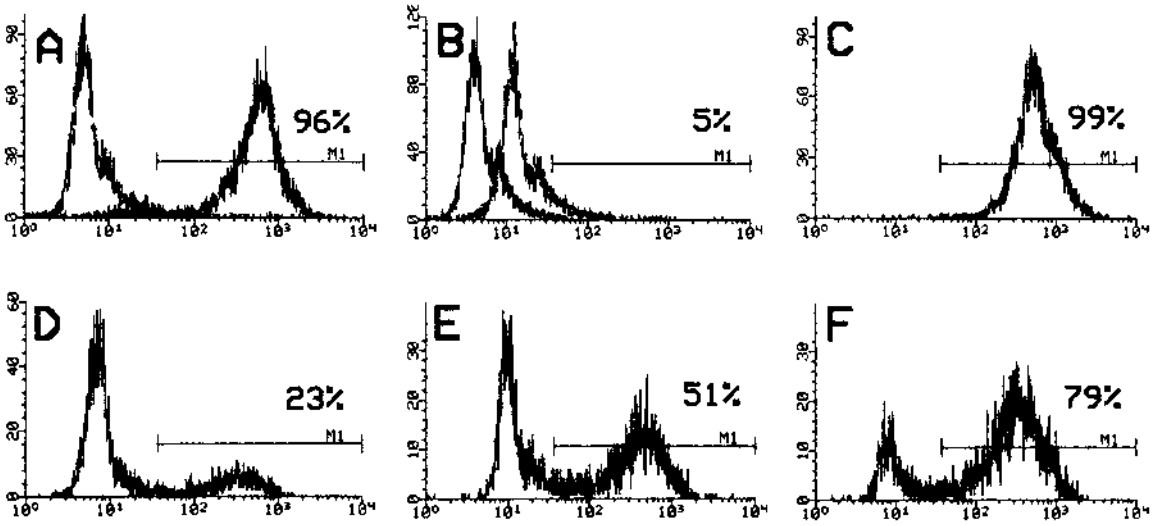

Fig. 1. Enumeration of erythocytes containing HbF (F cells). Circulating erythrocytes were stained for intracellular γ-globin chains as described in Materials and Methods. Six examples of flow cytometric histograms are shown. A: F cell enumeration in a sample of cord blood, with 96% of cells staining positively for HbF. The isotype control antibody also is shown. B: F cell enumeration on erythrocytes from a normal donor, with only a small proportion of positive cells. C: A patient with HPFH has >99% F cells. D: A child with HbSS and 23% F cells. E: A child with 51% F cells. F: A child with 79% F cells. In each patient with sickle cell disease, the quantitation of F cells is straightforward, due to the clear separation between erythrocytes that contain fetal hemoglobin and those that do not.

TABLE I. Clinical and Laboratory Parameters for 242 Children With Sickle Cell Disease*

Parameter	Hb S/HPFH	Hb SS	Hb S/β^0 thalassemia	Hb S/β^+ thalassemia	Hb SC
Total patients	8	130	7	23	74
Males	5	73	3	12	40
Females	3	57	4	11	34
Age (years)	10.1 ± 7.8	8.7 ± 4.8	6.6 ± 3.6	8.4 ± 4.7	8.4 ± 4.7
F cells					
%	98.9 ± 0.9	55.9 ± 19.9	61.6 ± 27.3	51.3 ± 14.0	27.0 ± 15.8
Range	96.9 – 99.8	16.7 – 94.4	23.2 – 91.4	29.5 – 85.2	5.1 – 72.7
Hb F					
%	31.7 ± 3.0	10.4 ± 6.3	15.4 ± 10.3	9.2 ± 5.0	3.3 ± 2.9
Range	26.8–37.4	1.5–28.9	3.4–28.0	3.2–21.8	0.5–14.6
Hgb (g/dL)	12.8 ± 1.4	8.0 ± 1.0	8.4 ± 1.7	10.7 ± 1.1	10.8 ± 1.1
Reticulocytes (%)	1.8 ± 0.4	14.3 ± 6.4	7.0 ± 2.6	2.2 ± 1.1	3.9 ± 2.5
Mean corpuscular volume (fL)	78 ± 9	85 ± 8	69 ± 4	69 ± 5	73 ± 6
Mean corpuscular Hgb (pg)	26.8 ± 2.6	29.0 ± 3.2	22.6 ± 1.9	23.2 ± 2.1	25.6 ± 2.1
Mean corpuscular Hgb concentration (g/dL)	34.7 ± 1.1	34.0 ± 1.2	32.7 ± 1.2	33.7 ± 0.8	35.1 ± 1.0
White blood cells ($\times 10^9$/L)	7.7 ± 2.5	12.9 ± 4.3	10.0 ± 2.8	7.8 ± 2.7	8.1 ± 2.3
Platelets ($\times 10^9$/L)	299 ± 138	494 ± 178	388 ± 234	326 ± 105	316 ± 149

*Values represent the mean ± 1 standard deviation for each group.

are summarized in Table I. Experimental values are from a single measurement, although 49 older children with serial measurements demonstrated a high degree of stability over time (0.8 ± 0.7% variability in % HbF, 4.9 ± 3.4% variability in % F cells). Eight patients with HbS/HPFH had virtually 100% F cells, which was significantly higher than the mean values for children with HbSS, HbS/β^0 thalassemia, or HbS/β^+ thalassemia (55.9, 61.6, and 51.3%, respectively; $P < 0.001$ for each comparison). There were no significant differences in the mean F cell enumeration among these latter three groups, and their ranges of % F cell values were similar. Children with HbSC had even fewer F cells than any other group of children (27.0%, $P < 0.001$ for each comparison).

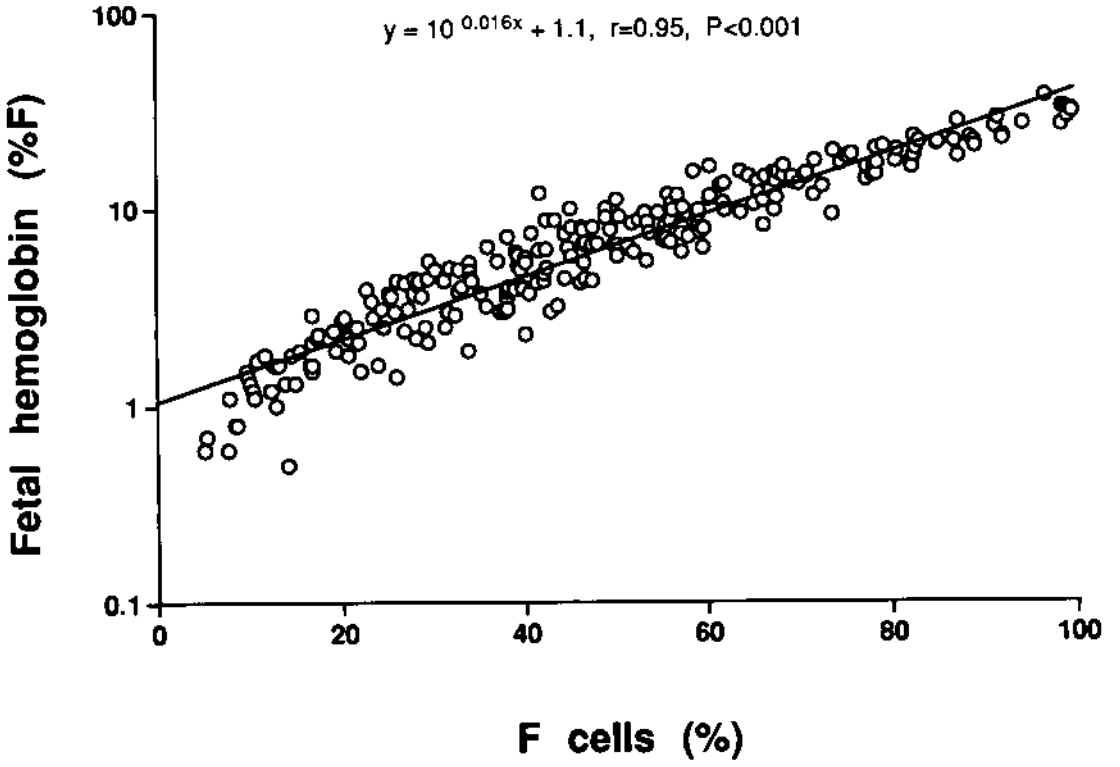

Fig. 2. Comparison of % F cells with % HbF for 242 pediatric patients with sickle cell disease, including 130 with HbSS, 74 with HbSC, 23 with HbS/β$^+$ thalassemia, 8 with HbS/HPFH, and 7 with HbS/β$^\circ$ thalassemia. The F cell enumeration was performed as described in Materials and Methods, and % HbF was quantitated by alkali denaturation. The % F cells was significantly correlated with log (% HbF) (r = 0.95, $P < 0.001$).

Quantitation of fetal hemoglobin revealed a similar pattern (Table I). The children with HbS/HPFH had a mean fetal hemoglobin concentration of 31.7%, which was significantly higher than the mean % HbF for children with HbSS, HbS/β^0 thalassemia, or HbS/β$^+$ thalassemia (10.4, 15.4, and 9.2%, respectively; $P < 0.001$ for each comparison). Children with HbSC had a significantly lower mean % HbF (3.3%; $P < 0.001$ for each comparison).

Additional hematologic parameters are also listed for each subset of patients, including the hemoglobin concentration, reticulocyte count as determined by an automated technique, red cell indices, white blood cell count, and platelet count (Table I).

Comparison of % F Cells With Other Laboratory Parameters

The percentage of F cells was compared with the other laboratory parameters listed in Table I. A highly significant correlation was found between the % F cells and the % HbF. For the entire patient population, the % HbF increased logarithmically with a linear increase in the % F cells (Fig. 2, r = 0.95, $P < 0.001$). This relationship was next analyzed according to hemoglobin genotype. Within each group, a similar strong correlation was found between % F cells and log (% HbF) (Fig. 3): for the 130 children with HbSS, r = 0.94 ($P < 0.001$); for the 74 children in the HbSC group, r = 0.89 ($P < 0.001$); and for the combined HbS/β thalassemia group (7 with HbS/β^0 thalassemia and 23 with HbS/β$^+$ thalassemia), r = 0.95 ($P < 0.001$).

For the children with HbSS, a significant correlation was also found between the % F cell value and the hemoglobin concentration (r = 0.54; $P < 0.001$). For each of the other subgroups, no statistically significant correlation was identified between these two variables.

DISCUSSION

The tendency toward intracellular HbS polymerization is probably the most important determinant of SCD pathophysiology [27,28]. Theoretically, therefore, the presence of intracellular HbF is advantageous for several reasons. First, an increased amount of HbF within the erythrocyte decreases the proportion of HbS within that cell. As a consequence, fewer HbS tetramers form, and less hemoglobin polymerization occurs within the cell [29]. In addition, HbF does not co-polymerize well with HbS, thereby

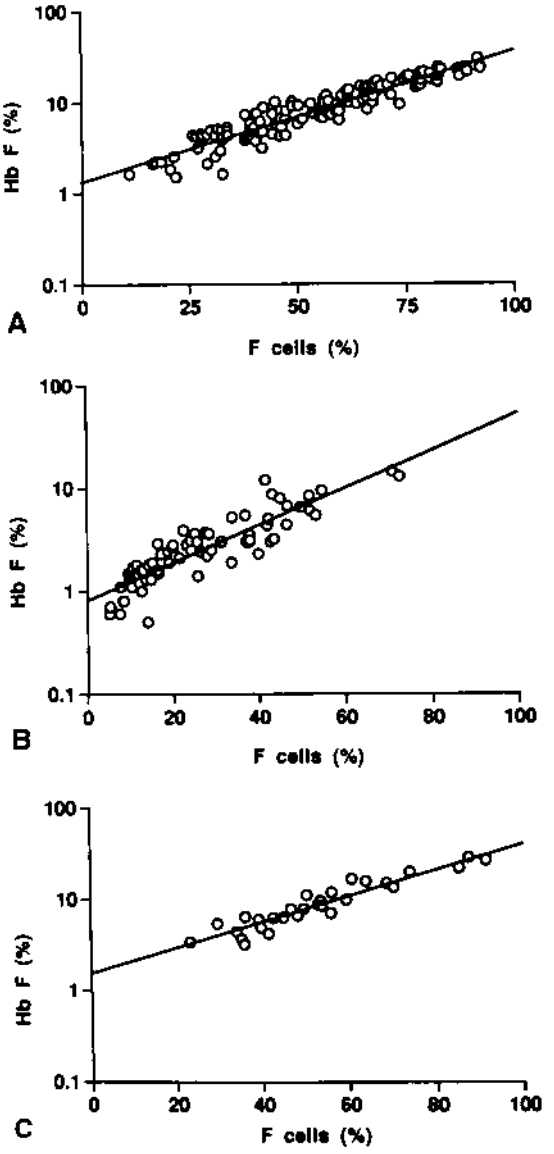

Fig. 3. Comparison of % F cells with % HbF according to hemoglobin genotype. A: Data for children with HbSS (n = 130, r = 0.94, $P < 0.001$). B: Patients with HbSC (n = 74, r = 0.89, $P < 0.001$). C: Correlation for children with HbS/β thalassemia (n = 23 with HbS/β^+ thalassemia and n = 7 with HbS/β^0 thalassemia; r = 0.95, $P < 0.001$).

increasing the solubility of intracellular HbS [30–33]. Finally, a greater amount of HbF within a cell increases the likelihood of the inclusion of γ-globin chains within the hemoglobin tetramers. These hybrid hemoglobin tetramers hinder HbS polymerization and may be completely excluded from HbS polymers [29,32,34].

Over 30 years ago, Shepard and colleagues [35] used a modification of the Kleihauer-Betke stain to determine that patients with SCD had a variable number of F cells. Analyzing the peripheral blood of eight patients, they noted an unequal distribution of fetal hemaglobin within the circulating erythrocytes. To date, measurement of the % F cells in large numbers of patients with SCD has not been reported. We used a quantitative assay for F cell enumeration in our study and found that children with HbSS, HbS/β^0 thalassemia, or HbS/β^+ thalassemia had very similar % F cell values, with a mean of approximately 50–60% F cells for each group and a range of F cell values from approximately 20 to 90%. In contrast, children with HbSC had significantly fewer F cells on average (27.0%) and also a lower mean % HbF (3.3%).

The increase in F cells in SCD patients has been attributed to an increased rate of erythropoiesis [12,14]; this hypothesis is supported by the reports of increased % F cells following recovery from phlebotomy, transient erythroblastopenia [36], or glucose-6-phosphate dehydrogenase (G6PD)-deficient hemolysis [37]. Perhaps children with HbSS have significantly higher % F cells than children with HbSC due to a higher rate of erythropoiesis, as measured by their baseline hemoglobin concentration and reticulocyte counts. However, children with HbS/β^+ thalassemia had average hemoglobin and reticulocyte counts very similar to those of the HbSC patients (Table I) but had significantly higher % F cells (51.3% vs. 27.0%; $P < 0.001$). Therefore, the % F cells cannot be based solely on the rate of erythropoiesis. A variety of factors appear to influence the % F cells, including an incompletely understood X-linked F cell production locus [20,22].

Perhaps the most important observation from our analysis was the highly significant logarithmic correlation between the % F cells and the % HbF, which has not been previously described. In an older study, which used a less accurate method of F cell enumeration, Wood et al. [14] found a linear correlation between these two parameters, but the % HbF ranged from only 0 to 3% in their population. Our patients had a much broader range and higher % HbF values, which allowed us to identify the logarithmic correlation (Figs. 2, 3). An important implication of this observation is that the average amount of HbF per F cell varies among individual patients with SCD. For example, if each F cell had a fixed amount of intracellular HbF, then a patient with 60% F cells should have 3 times the % HbF as a patient with only 20% F cells. This would be the result of a linear correlation between the two parameters. The logarithmic correlation is illustrated by the observation that a threefold difference in % F cells (60% vs. 20%) is associated with a fivefold difference in % HbF (10% vs. 2%). This logarithmic correlation was observed for all subsets of SCD patients, but the mechanisms responsible for this highly significant logarithmic association are unknown. It is possible that the % F cells is genetically predetermined in a given patient and that the amount of HbF per F cell is somehow regulated by the total number of F cells within the erythroid progenitor pool.

The logarithmic association between % F cells and % HbF also has important implications for the pharmacologic modification of HbF in patients with SCD. Hydroxyurea is currently being used to increase HbF production in SCD and increases both the % F cells and the % HbF [38–40]. Charache et al. [39] observed a mean increase in HbF of 11% after treatment with hydroxyurea. For a patient starting with 2% HbF, this increase in HbF to 13% would increase the % F cells from approximately 20 to 70%. In contrast, an identical incremental increase in % HbF from 10 to 21% would correspond to an increase in % F cells from 60 to 80%. In terms of increasing the number of F cells that could resist intracellular HbS polymerization, pharmacologic manipulation might be more beneficial for patients who begin with lower % HbF levels. However, patients who being with higher % HbF levels would have a greater increase in the amount of HbF per F cell, which would also presumably provide a clinical benefit. Quantitative measurement of both % HbF and % F cells should be performed in SCD patients receiving agents such as hydroxyurea and should be correlated with changes in other laboratory or clinical parameters. Greater experience with these pharmacologic agents will determine which patients benefit most.

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